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Kdo Hydrolase Is Required for *Francisella tularensis* Virulence and Evasion of TLR2-Mediated Innate Immunity

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ABSTRACT The highly virulent *Francisella tularensis* subsp. *tularensis* has been classified as a category A bioterrorism agent. A live vaccine strain (LVS) has been developed but remains unlicensed in the United States because of an incomplete understanding of its attenuation. Lipopolysaccharide (LPS) modification is a common strategy employed by bacterial pathogens to avoid innate immunity. A novel modification enzyme has recently been identified in *F. tularensis* and *Helicobacter pylori*. This enzyme, a two-component Kdo (3-deoxy-D-manno-octulosonic acid) hydrolase, catalyzes the removal of a side chain Kdo sugar from LPS precursors. The biological significance of this modification has not yet been studied. To address the role of the two-component Kdo hydrolase KdhAB in *F. tularensis* pathogenesis, a $\Delta kdhAB$ deletion mutant was constructed from the LVS strain. In intranasal infection of mice, the $\Delta kdhAB$ mutant strain had a 50% lethal dose (LD_{50}) $2 \log_{10}$ units higher than that of the parental LVS strain. The levels of the proinflammatory cytokines tumor necrosis factor alpha (TNF- α) and interleukin-1 β (IL-1 β) in bronchoalveolar lavage fluid were significantly higher (2-fold) in mice infected with the $\Delta kdhAB$ mutant than in mice infected with LVS. *In vitro* stimulation of bone marrow-derived macrophages with the $\Delta kdhAB$ mutant induced higher levels of TNF- α and IL-1 β in a TLR2-dependent manner. In addition, TLR2 $^{-/-}$ mice were more susceptible than wild-type mice to $\Delta kdhAB$ bacterial infection. Finally, immunization of mice with $\Delta kdhAB$ bacteria elicited a high level of protection against the highly virulent *F. tularensis* subsp. *tularensis* strain Schu S4. These findings suggest an important role for the *Francisella* Kdo hydrolase system in virulence and offer a novel mutant as a candidate vaccine.

IMPORTANCE The first line of defense against a bacterial pathogen is innate immunity, which slows the progress of infection and allows time for adaptive immunity to develop. Some bacterial pathogens, such as *Francisella tularensis*, suppress the early innate immune response, killing the host before adaptive immunity can mature. To avoid an innate immune response, *F. tularensis* enzymatically modifies its lipopolysaccharide (LPS). A novel LPS modification—Kdo (3-deoxy-D-manno-octulosonic acid) saccharide removal—has recently been reported in *F. tularensis*. We found that the $\Delta kdhAB$ mutant was significantly attenuated in mice. Additionally, the mutant strain induced an early innate immune response in mice both *in vitro* and *in vivo*. Immunization of mice with this mutant provided protection against the highly virulent *F. tularensis* strain Schu S4. Thus, our study has identified a novel LPS modification important for microbial virulence. A mutant lacking this modification may be used as a live attenuated vaccine against tularemia.

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Francisella tularensis, one of the deadliest respiratory pathogens in existence, is considered a potential biological weapon because it is readily aerosolized and exhibits a high degree of infectivity and lethality in humans. An attenuated live vaccine strain (LVS) has been developed but has not been licensed as a vaccine because of an incomplete understanding of the basis for its attenuated virulence and associated side effects. There has been a widespread research effort aimed at elucidating *Francisella* pathogenesis and identifying components for rational vaccine design. *F. tularensis* is a successful pathogen that can evade and suppress innate immune responses. After intranasal infection of mice, *F. tularensis* initially colonizes the lung, infecting several cell types but failing to stimulate proinflammatory cytokine production for

the first 2 to 3 days (1–5). This initial delay in inflammation contributes to the ability of *F. tularensis* to replicate in the lung, spread into systemic organs, and cause sepsis leading to the host's death (4). To suppress the early proinflammatory cytokine response, *F. tularensis* employs a variety of strategies, such as avoiding disclosure of itself to the innate immune system (6, 7).

Lipopolysaccharide (LPS) of *F. tularensis* plays a significant role in evasion of host immune responses (8–11). LPS, the major component of Gram-negative bacterial outer membranes (12, 13), itself has three components: (i) lipid A, a glucosamine-based glycolipid; (ii) a core oligosaccharide ketosidically linked to lipid A by an eight-carbon sugar, 3-deoxy-D-manno-octulosonic acid (Kdo); and (iii) the O-antigen polysaccharide comprising multi-

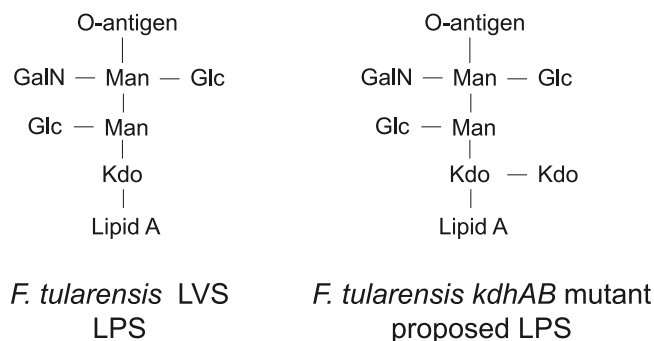


FIG 1 LPSs from *F. tularensis* LVS and $\Delta kdhAB$ mutant strain differ from each other by a side chain Kdo. The structure of core oligosaccharide synthesized by LVS (55) and the proposed structure of core oligosaccharide synthesized by the $\Delta kdhAB$ mutant, as determined by HPAEC (14), are shown.

ple repeating units of a complex tetrasaccharide. The lipid A of many bacterial species such as *Escherichia coli* is a powerful stimulant of Toll-like receptor 4 (TLR4)-mediated innate immune responses. In contrast, *F. tularensis* lipid A triggers no TLR-mediated innate response because of a number of key structural modifications (9); specifically, despite initial similarity to *E. coli* lipid A, the lipid A precursor of *F. tularensis* undergoes dephosphorylation, deacylation, and carbohydrate incorporation that render the molecule undetectable by the TLR4 receptor. Differences in the length of the fatty acid chain of lipid A (16 to 18 carbons for *F. tularensis* as opposed to 12 to 14 carbons for *E. coli*) further enhance the ability of *F. tularensis* to evade TLR4-mediated innate immunity. These modifications contribute to bacterial virulence (8, 10).

A novel LPS-modifying two-component KdhAB Kdo hydrolase enzyme complex has recently been identified in *F. tularensis* (14, 15). A core LPS precursor containing two Kdo residues is first synthesized. One Kdo in the backbone of the core polysaccharide is ketosidically linked to lipid A and glycosidically linked to the remainder of the core polysaccharide, and the second Kdo residue is glycosidically attached as a side chain. KdhAB removes the side chain Kdo, leaving an LPS molecule containing only one Kdo residue (Fig. 1) (14–16). The KdhAB Kdo hydrolase is a two-component enzyme composed of a sialidase-like protein (the catalytic subunit) and a small inner membrane protein (perhaps a membrane-anchoring subunit) (15). This novel enzyme complex has been reported in two other pathogens, *Helicobacter pylori* and *Legionella pneumophila* (14, 17, 18). Its biological significance—in particular, its role in pathogenicity—has not yet been investigated. Here, we show that the *F. tularensis* $\Delta kdhAB$ LVS mutant is attenuated in a mouse model of infection and stimulates—in a TLR2-dependent manner—a stronger proinflammatory cytokine response than the parental LVS. Furthermore, immunization of mice with the $\Delta kdhAB$ mutant provides significant protection against fully virulent *F. tularensis* type A strain Schu S4.

RESULTS

The *F. tularensis* LVS $\Delta kdhAB$ mutant is attenuated in mice. To fully evaluate the importance of the KdhAB Kdo hydrolase in the virulence of *F. tularensis* LVS, *kdhA* and *kdhB* genes were deleted by homologous recombination in an LVS background, giving rise to the $\Delta kdhAB$ mutant. Previously described analysis by high-performance anion-exchange chromatography (HPAEC) (14)

confirmed that, unlike the LPS of parental LVS, which has a single Kdo residue, the LPS of the $\Delta kdhAB$ mutant has two Kdo residues (Fig. 1; see Table S1 in the supplemental material).

To evaluate the role of Kdo hydrolase activity in *F. tularensis* virulence, we infected BALB/cByJ mice with graded doses of LVS or the $\Delta kdhAB$ mutant via the intranasal route. All mice challenged with 10^3 CFU of LVS succumbed to the infection within 9 days (Fig. 2A). In contrast, all mice challenged with a similar dose of the $\Delta kdhAB$ mutant survived (Fig. 2A). At a dose of 10^5 CFU of the $\Delta kdhAB$ mutant, 17% of the infected mice survived (Fig. 2A).

The observed phenotypes in the $\Delta kdhAB$ mutant were successfully complemented with the *pkdhAB* plasmid carrying the *kdhA* and *kdhB* genes. LPS analysis showed that the complemented strain had only a single Kdo residue in its core LPS (see Table S1 in the supplemental material). In addition, complementation of $\Delta kdhAB$ restored virulence to the wild-type (WT) level. The survival rates of BALB/cByJ mice (10 mice in each group) 28 days after an intranasal challenge with 5×10^3 *F. tularensis* LVS or a $\Delta kdhAB$ mutant harboring either a vector control (pFNLT6) or a plasmid carrying *kdhAB* genes (*pkdhAB*) were as follows: 0% for mice infected with LVS, 100% for mice infected with the $\Delta kdhAB$ mutant carrying pFNLT6, and 20% for mice infected with the $\Delta kdhAB$ mutant carrying *pkdhAB*. These data indicate that Kdo hydrolase activity is important for the virulence of *F. tularensis* LVS.

We also examined the ability of the $\Delta kdhAB$ mutant to colonize the lung and to disseminate into other organs after intranasal challenge of mice. Six days after infection with 10^3 bacteria, both parental LVS and its $\Delta kdhAB$ mutant had reached a similar level of colonization in the lungs, with counts of 3×10^6 CFU/g and 10^7 CFU/g, respectively (Fig. 2B). However, the numbers of $\Delta kdhAB$ mutant bacteria in spleen, liver, and blood were 1.5 to 3 logarithmic units lower than the numbers of LVS bacteria (Fig. 2B) (Student's *t* test, $P < 0.05$). Overall, these results indicate that the $\Delta kdhAB$ mutant is deficient in its ability to disseminate systemically.

To determine whether the observed defect in dissemination is related to bacterial growth kinetics, growth rates were compared under different conditions. There was no difference in growth between LVS and the $\Delta kdhAB$ mutant when bacteria were cultured in broth medium or in J774A.1 macrophage-like and MH-S alveolar macrophage-like cell lines (see Fig. S1A and S1B in the supplemental material). Moreover, the $\Delta kdhAB$ mutant was not sensitive to complement-mediated killing (Fig. S1C). Therefore, the defect of the $\Delta kdhAB$ mutant in dissemination to systemic organs was not due to a growth defect or to serum sensitivity.

The cytokine response to the *F. tularensis* LVS $\Delta kdhAB$ mutant is enhanced. Within the first day after infection, mutants of *F. tularensis* that cannot modify their lipid A elicit a stronger proinflammatory response than parental LVS did (8, 19). Since the KdhAB hydrolase is involved in the modification of the LPS core oligosaccharide but not in the modification of lipid A, we compared the proinflammatory responses to LVS and the $\Delta kdhAB$ mutant. To this end, mice were challenged intranasally with 10^3 CFU of either LVS or the $\Delta kdhAB$ mutant, and the levels of tumor necrosis factor alpha (TNF- α) and interleukin-1 β (IL-1 β) in samples of bronchoalveolar lavage (BAL) fluid were determined in an enzyme-linked immunosorbent assay (ELISA). Consistent with previous reports (6), the levels of TNF- α and IL-1 β in

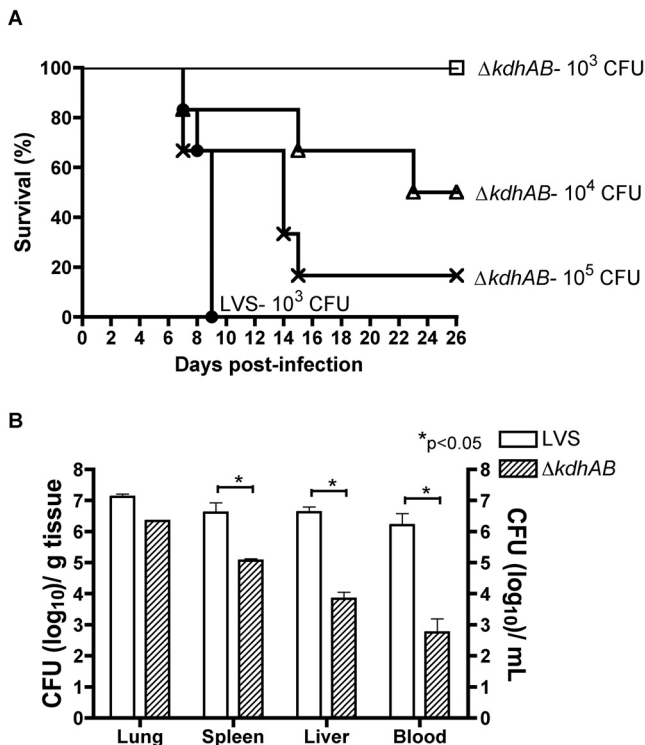


FIG 2 The virulence of the *F. tularensis* LVS $\Delta kdhAB$ mutant strain is attenuated in mice. (A) Survival of mice after *F. tularensis* intranasal challenge. BALB/cByJ mice ($n = 6$) were challenged intranasally with LVS (10^3 CFU) or $\Delta kdhAB$ bacteria (10^3 , 10^4 , or 10^5 CFU). (B) Bacterial burden in lung, spleen, liver, and blood of mice 6 days after infection. BALB/cByJ mice ($n = 3$) were challenged intranasally with LVS bacteria (10^3 CFU) or $\Delta kdhAB$ bacteria (10^3 CFU).

BAL fluid 24 h after inoculation of LVS bacteria were not different from those in control mice given phosphate-buffered saline (PBS) (Fig. 3A, left panel). In contrast, the levels of TNF- α and IL-1 β were significantly higher (~2-fold) in mice infected by the $\Delta kdhAB$ mutant than in mice infected by LVS or control mice given PBS (Fig. 3A, left panel). The stronger proinflammatory response was not due to differences in bacterial burdens, as the numbers of $\Delta kdhAB$ and LVS bacteria in the lungs were similar (Fig. 3A, right panel). Therefore, in contrast to LVS, the $\Delta kdhAB$ mutant elicited an early proinflammatory response that was detected 24 h after infection.

To test whether the *in vivo* cytokine response also differs in *ex vivo* macrophages, bone marrow-derived macrophages (BMMs) from C57BL/6J mice were infected at different multiplicities of infection (MOIs) with either LVS or the $\Delta kdhAB$ mutant. Supernatants were collected after 24 h, and secretion of TNF- α and IL-1 β was measured by ELISA. At all MOIs tested, TNF- α secretion by BMMs stimulated by the $\Delta kdhAB$ mutant was higher by a factor of at least 2 than that by BMMs stimulated by LVS (Fig. 3B, left panel). Results were similar for IL-1 β secretion (Fig. 3B, right panel). Consistent with the cytokine secretion profile, the expression of TNF- α and IL-1 β genes was higher in $\Delta kdhAB$ mutant-stimulated BMMs than in LVS-stimulated BMMs (Fig. 3C).

The *F. tularensis* LVS $\Delta kdhAB$ mutant activates the TLR2 signaling pathway. To investigate the basis for the enhanced innate immune response to the $\Delta kdhAB$ mutant, we assessed the re-

sponses of murine macrophage-like cell lines in which various TLR signaling pathways were inactivated. Cells were infected with either LVS or the $\Delta kdhAB$ mutant at an MOI of 100 and lysed 24 h later for measurement of IL-1 β . Cell activation was calculated as the IL-1 β ratio in infected cells versus uninfected cells. LVS bacteria elicited a proinflammatory response in WT, Unc93B $^{-/-}$, and TLR4 $^{-/-}$ cells, but not in MyD88 $^{-/-}$ or TLR2 $^{-/-}$ cells (Fig. 4A). These data are in agreement with previous reports that the innate immune response to LVS is mediated through TLR2 signaling (3, 20). Interestingly, the $\Delta kdhAB$ mutant showed a similar pattern of activation, a result indicating that TLR2—but not TLR3, -4, -7, or -9—mediates the innate immune response to $\Delta kdhAB$ mutant bacteria. Moreover, the proinflammatory response was significantly greater in $\Delta kdhAB$ mutant-infected cells than in LVS-infected cells (Fig. 4A). Control experiments confirmed that WT cells and knockout cells for Unc93B (required for TLR3, TLR7, and TLR9 signaling), MyD88, TLR4, and TLR2 responded to various TLR agonists as expected (data not shown).

To assess whether $\Delta kdhAB$ mutant-mediated enhancement of the proinflammatory cytokine response is likely to be due to an increase in nuclear factor κ B (NF- κ B) activation, we used HEK293 cells expressing TLR2 (HEK-TLR2) or TLR4 and MD2 (HEK-TLR4/MD2). The stable cell lines were transiently transfected with the reporter plasmid pNF κ B-luc, which expresses a firefly luciferase gene under control of an NF- κ B promoter, and were incubated with LVS or $\Delta kdhAB$ organisms. Reporter activity in HEK-TLR4/MD2 cells incubated with bacteria of either strain did not differ (Fig. 4B). On the other hand, reporter activity increased slightly but significantly in HEK-TLR2 cells stimulated with the $\Delta kdhAB$ mutant over that in HEK-TLR2 cells stimulated with LVS ($P < 0.05$) (Fig. 4B). Control experiments using *E. coli* LPS and Pam₃CSK, known TLR4 and TLR2 antagonists, respectively, showed high NF- κ B reporter activity as expected (Fig. 4B).

Mechanistic basis for activation of TLR2 by the $\Delta kdhAB$ mutant. One possible explanation for the increase in TLR2-mediated NF- κ B activity is that, unlike WT LVS LPS, the mutant LPS produced by the $\Delta kdhAB$ mutant is a TLR2 agonist (9). To test this hypothesis, we assessed purified LPS preparations from each strain for their ability to activate NF- κ B in HEK-TLR2 cells. LPS from LVS and the $\Delta kdhAB$ mutant showed similar levels of NF- κ B reporter activity, which was also similar to that elicited by whole LVS organisms (Fig. 4B). Additionally, BMMs did not respond to LPS from either LVS or the $\Delta kdhAB$ mutant by secretion of TNF- α and IL-1 β when the purified LPS was tested at concentrations of 50, 250, 1,250, and 10,000 ng/ml (see Fig. S2 in the supplemental material). Taken together, these results show that the $\Delta kdhAB$ mutant elicits stronger NF- κ B activation in a TLR2-dependent manner, with a consequently greater proinflammatory response. However, the greater proinflammatory response induced by the mutant LPS probably is not due to activation of TLR2 by the mutant LPS *per se*, as the LPS of the $\Delta kdhAB$ mutant alone does not trigger TLR2 signaling *in vitro*.

To further investigate the mechanism for enhanced TLR2 signaling by the $\Delta kdhAB$ mutant, we evaluated bacterial membrane integrity. A leaky cell membrane caused by $\Delta kdhAB$ mutation would potentially release TLR2 ligands into the culture supernatant to be detected by host cells. Thus, the growth behaviors of LVS and the $\Delta kdhAB$ mutant were tested in the presence of SDS and EDTA, agents that inhibit growth of bacterial cells with defective outer membranes. The similar growth characteristics of the two

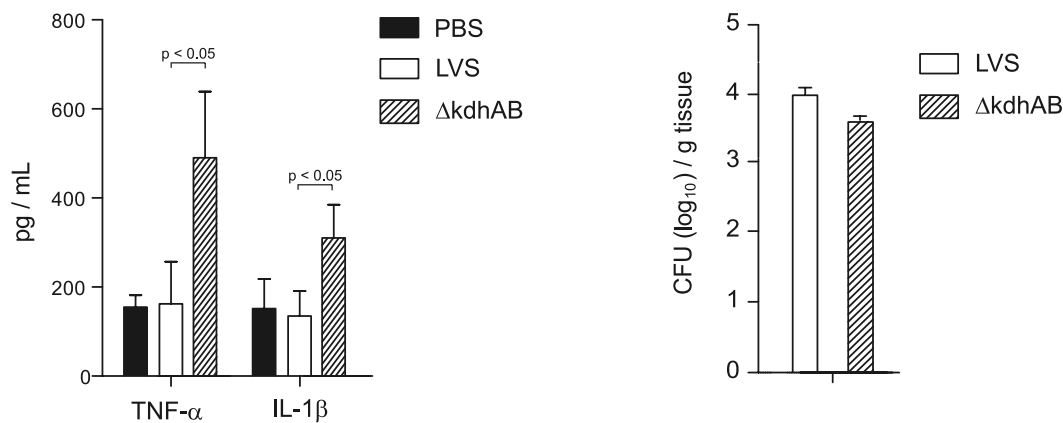
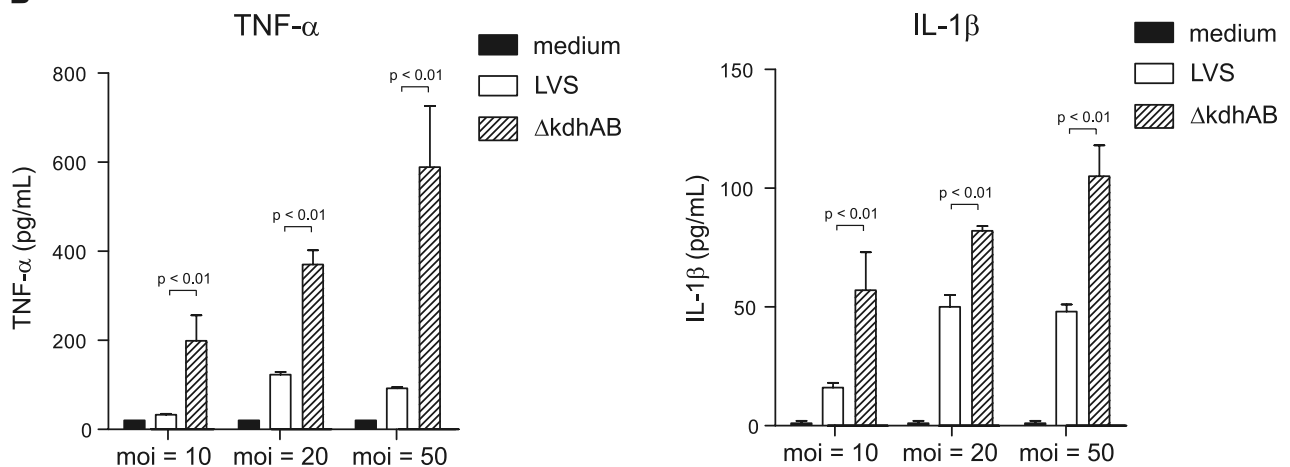
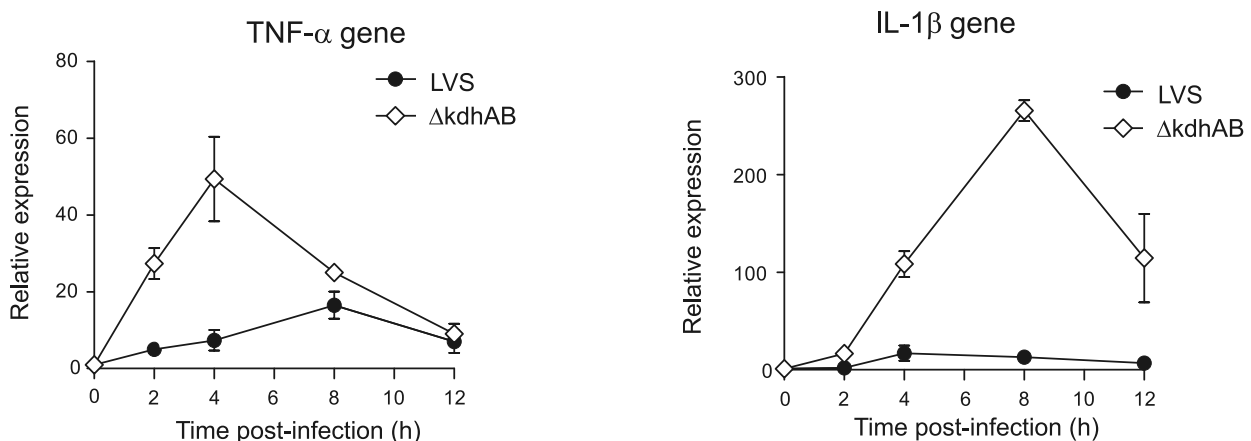
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FIG 3 Enhanced proinflammatory cytokine response to the *F. tularensis* LVS $\Delta kdhAB$ mutant strain. (A) BALB/cByJ mice ($n = 3$) were challenged intranasally with LVS bacteria (10^3 CFU), $\Delta kdhAB$ bacteria (10^3 CFU), or PBS (control). BAL fluid was collected 24 h after infection, and the levels of TNF- α and IL-1 β were measured by ELISA (left panel). The bacterial burden in lung was measured 24 h after infection (right panel). (B) Levels of TNF- α and IL-1 β released by *ex vivo* BMMs after stimulation with LVS or $\Delta kdhAB$ bacteria (MOI of 10, 20, or 50). Culture supernatants were analyzed by ELISA 24 h after infection. (C) TNF- α and IL-1 β gene expression in BMMs infected with LVS or $\Delta kdhAB$ bacteria (MOI of 10). Gene expression was measured by real-time PCR, normalized with RP II gene expression, and reported as relative expression compared with that in uninfected macrophages. All data are presented as mean plus SD (error bars) values ($n = 3$).

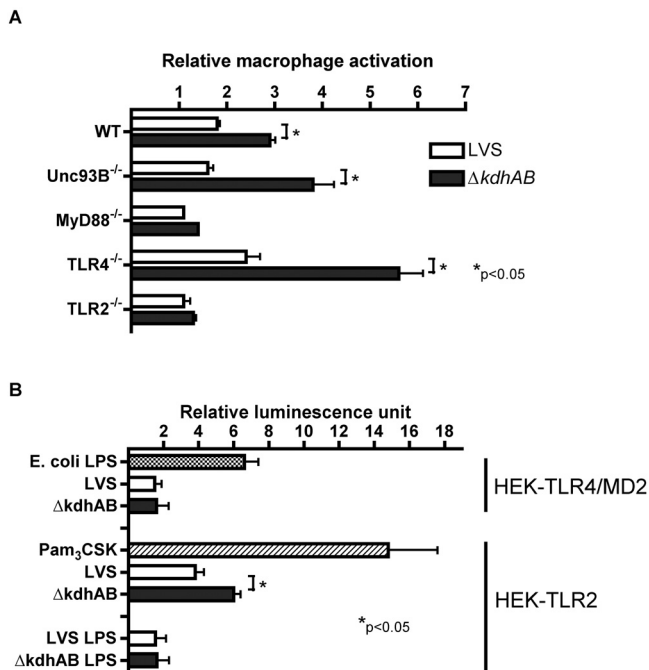


FIG 4 Bacteria of the *F. tularensis* LVS $\Delta kdhAB$ mutant strain activate NF- κ B through the TLR2 signaling pathway. (A) Macrophage-like cell lines were stimulated with LVS or $\Delta kdhAB$ bacteria for 2 h (MOI = 100). The IL-1 β level in cell lysates was assessed by ELISA. Measurements were expressed as fold induction in infected versus uninfected cells (mean plus SD; $n = 3$). (B) HEK293 cells stably expressing TLR2 or TLR4/MD2 were transiently transfected with an NF- κ B luciferase reporter plasmid and stimulated for 24 h with LVS or $\Delta kdhAB$ bacteria (MOI = 20) or with Pam₃CSK (1 μ g/ml) or *E. coli* LPS (100 ng/ml) as positive controls. Purified LPSs from LVS or $\Delta kdhAB$ bacteria (5 μ g/ml) were tested in a mixture with soluble CD14 recombinant protein (5 μ g/ml). The data are reported as relative luciferase units and represent fold induction of luciferase activity in the cell lysate compared with that in unstimulated cells (mean plus SD; $n = 3$).

strains (Fig. 5A) strongly supported the notion that the $\Delta kdhAB$ mutation does not compromise cell membrane integrity. In an alternative approach, we tested the filtered supernatants of LVS or $\Delta kdhAB$ cultures for their ability to activate BMMs *in vitro*. The culture supernatants were concentrated by Centricon filter devices and incubated with BMMs for 24 h. Macrophage activation was measured by the release of TNF- α and IL-6. While the addition of LPS or PAM₃CSK (as positive controls) to the BMMs did result in significant activation of the cells, little activation was seen after the addition of the LVS- or $\Delta kdhAB$ mutant-conditioned media (see Fig. S3 in the supplemental material). The similarly low levels of TNF- α and IL-6 for both LVS and $\Delta kdhAB$ organisms suggested that there is no leakage of potential TLR2 ligands due to the $\Delta kdhAB$ mutation.

Unable to detect a leaky-membrane phenotype, we next investigated changes in bacterial LPS and cell-surface protein availability. It has been proposed that fully formed LPS on the bacterial surface plays a vital role in preventing the binding of serum complement, antibodies, and receptors of the innate immune system (21–24). Toward this end, the analysis of total LPS level and a characteristic LPS banding pattern showed no difference between LVS and the $\Delta kdhAB$ mutant (Fig. 6A). Additionally, matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) analysis of lipid A indicated that LVS and the $\Delta kdhAB$ mutant

have similar lipid A mass values (Fig. 6B). Although the $\Delta kdhAB$ mutation does not affect the total LPS level or the characteristic banding pattern of LPS, we thought it is possible that the additional Kdo—a negatively charged sugar—in the $\Delta kdhAB$ mutant alters the conformation of LPS molecules, thereby making other surface constituents more accessible to TLR2 receptors. To test this hypothesis, we evaluated the binding of FopA antibodies to the FopA surface protein *in vitro*. Live bacteria were incubated with mouse antibodies to FopA, and surface-bound antibodies were analyzed by Western blotting. Consistent with a previous study (24), fully formed LPS significantly reduced the binding of antibodies to the LVS bacterial surface, whereas $\Delta wbtA$ mutant LPS, which is devoid of O polysaccharide, did not (Fig. 5B). Strikingly, substantial binding of FopA antibodies to the $\Delta kdhAB$ mutant bacterial surface was found, and this result suggested that a TLR2 ligand present on the bacterial cell surface would be potentially exposed for detection by host immune-cell TLR2 receptors. The accessibility of surface proteins might well explain the enhanced TLR2 signaling activity caused by the $\Delta kdhAB$ mutation.

Biological impact of TLR2 activation by the $\Delta kdhAB$ mutant.

We next addressed the biological importance of the TLR2-mediated response to $\Delta kdhAB$ bacteria in a mouse infection model. WT C57BL/6, TLR2^{-/-}, and TLR4^{-/-} mice were infected with various doses of the $\Delta kdhAB$ mutant via the intranasal route and monitored for survival. Most strikingly, at a dose of 10⁴ CFU, all WT and TLR4^{-/-} mice survived, while no TLR2^{-/-} mice survived (Table 1). The results suggest that TLR2-mediated responses play a significant role in the survival of mice infected with the *F. tularensis* $\Delta kdhAB$ mutant strain. Thus, the association of KdhAB Kdo hydrolase with enhanced TLR2-mediated inflammation points to a critical role for this enzyme complex in the virulence of LVS.

Immunization with *F. tularensis* LVS $\Delta kdhAB$ mutant protects against the highly virulent *F. tularensis* Schu S4 strain. Immunization with LVS is protective against the fully virulent *F. tularensis* type A strain Schu S4 in a mouse model of infection, and this protection involves TLR2-dependent innate immunity (25–27). However, even in low numbers, LVS retains significant virulence in mice. Our findings that the $\Delta kdhAB$ mutant is further attenuated and elicits enhanced TLR2 signaling led us to investigate the protective capacity of this mutant strain as a live cell vaccine. To this end, BALB/cByJ mice were immunized with three intranasal doses of LVS, the $\Delta kdhAB$ mutant, or (as a control) PBS. Four weeks after the last dose, the mice were challenged via the intranasal route with either LVS at a dose of 10⁴ CFU (10 \times LD₅₀) or the Schu S4 strain at a dose of 10 CFU (10 \times LD₅₀). All mice that had been immunized with LVS survived LVS challenge, whereas all control mice that had received PBS succumbed within 9 days (Fig. 7A). The $\Delta kdhAB$ mutant was also fully protective against LVS challenge (Fig. 7A). Furthermore, as expected, LVS-immunized mice survived the fully virulent Schu S4 challenge, while all PBS-treated control mice succumbed within 7 days (Fig. 7B). Strikingly, mice immunized with the $\Delta kdhAB$ mutant were well protected; 90% of these mice survived an otherwise lethal challenge with Schu S4. Histologic examination of the livers from infected mice revealed that $\Delta kdhAB$ mutant-immunized mice had much less tissue necrosis after challenge with Schu S4 (Fig. 7C) than did PBS-treated control mice. The protection conferred by the $\Delta kdhAB$ mutant was also reflected by lower bacterial loads in the livers, spleens, and lungs from mutant-immunized

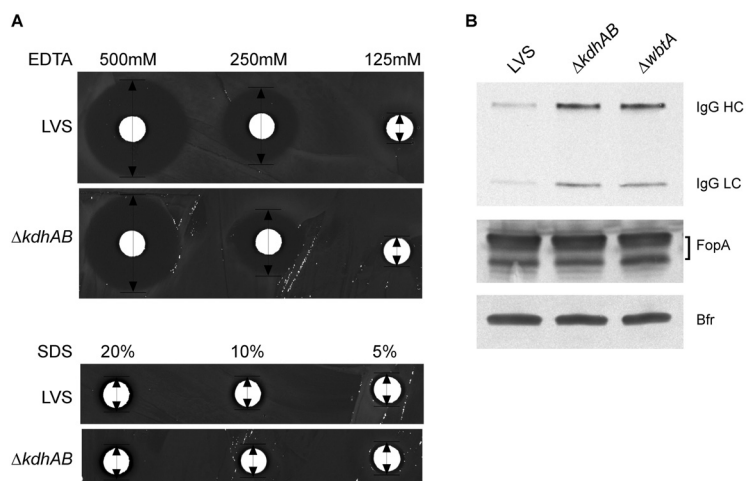


FIG 5 Mutation in *kdhAB* leads to enhanced accessibility of the bacterial surface. (A) Investigation of the leaky-membrane phenotype. Mid-logarithmic-phase cultures were spread onto CHAH plates, and a 10- μ l volume of SDS (20, 10, and 5%) or EDTA (500, 250 and 125 mM) was spotted onto filter discs. Images were captured after 48 h of bacterial growth. Black arrows indicate zone of inhibition around 6-mm filter discs. (B) *F. tularensis* LVS and $\Delta kdhAB$ and $\Delta wbtA$ mutant strains were tested for binding of mouse anti-FopA antibodies to their surface FopA proteins in an antigen accessibility assay. Bacteria—along with surface-bound FopA antibodies—were lysed, and proteins were resolved by 5 to 20% SDS-PAGE. Bound FopA antibodies were detected as IgG heavy chain (HC) and IgG light chain (LC). Total FopA and bacterioferritin (Bfr) protein levels are shown as protein loading controls.

mice (Fig. 7D) than in these tissues from PBS-treated control mice. The Schu S4 bacterial loads in organs were similar in LVS- and $\Delta kdhAB$ mutant-immunized mice. Finally, the levels of *Francisella*-specific IgG antibody were slightly higher in the sera of $\Delta kdhAB$ mutant-immunized mice compared to LVS-immunized mice (Fig. 7E).

DISCUSSION

Evasion of innate immunity is a key virulence factor for a number of pathogens, including *F. tularensis* (6, 7). To avoid detection by pattern recognition receptors such as TLRs, *F. tularensis* modifies its lipid A through the action of enzymes such as 4'-phosphatase, galactosamine transferase, and mannosyl transferase (8, 10). A novel LPS modification was hypothesized in *F. tularensis* and *H. pylori*: the removal of side chain Kdo from Kdo₂-lipid A precursors by a Kdo hydrolase (16, 17). Such a modification was recently confirmed, and the genes coding for a two-component Kdo hydrolase were identified in *F. tularensis* and *H. pylori* (14, 15, 17, 18). The biological significance of the Kdo hydrolase, which is also expressed by *L. pneumophila* (14), remained to be addressed. By screening an *F. tularensis* subsp. *novicida* transposon mutant library, Weiss and colleagues showed that a *kdhB* transposon mutant was attenuated in mice, but no further characterization has been reported (28). Here we demonstrate that the two-component Kdo hydrolase KdhAB is important in the pathogenicity of *F. tularensis* LVS bacteria and in the organism's avoidance of the TLR2-mediated immune response. We further show that a Kdo hydrolase-deficient mutant is protective against the fully virulent *F. tularensis* type A strain Schu S4. Such protective capacity is obviously a key feature to be hoped for in an effective vaccine strain.

The $\Delta kdhAB$ deletion mutant, unable to remove side chain

Kdo from its LPS (Fig. 1), was impaired in its ability to kill mice after intranasal challenge (Fig. 2). This attenuation was not due to a growth defect or to sensitivity to complement-mediated killing. In *H. pylori*, Kdo hydrolase inactivation has downstream effects on subsequent steps in LPS biosynthesis, reducing the efficiency of lipid A modification enzymes 4'-phosphatase and 3'-O-deacylase and the amount of fully extended O antigen (17). Both 4'-phosphatase and O antigen are required for full virulence of *F. tularensis* in mice (10, 19, 29). However, Kdo hydrolase inactivation in *F. tularensis* LVS did not affect lipid A or O-antigen biosynthesis (Fig. 6). Therefore, virulence attenuation was not related to a defect in 4'-phosphatase activity or O-antigen assembly.

The avoidance of the TLR response by modifications in lipid A structure has been described in a number of pathogens, including members of the genera *Salmonella*, *Yersinia*, *Pseudomonas*, and *Francisella* (8, 10, 30–32). These modifications include lipid A deacylation, dephosphorylation, and glycosylation. We thus hypothesized that *F. tularensis* removes side chain Kdo from LPS core oligosaccharide to avoid the triggering of innate immunity. In agreement with this hypothesis, the $\Delta kdhAB$ mutant stimulated a stronger TLR2-mediated proinflammatory response in macrophage monolayers than the parental LVS did (Fig. 3 and 4). *In vivo*, $\Delta kdhAB$ bacteria induced a proinflammatory response in the lungs 24 h after intranasal challenge, while LVS bacteria did not (Fig. 3A, left panel). Attenuation of the virulence of $\Delta kdhAB$ bacteria may be related to this early innate immune response. Indeed, several studies have shown that eliciting an early inflammatory response in mice by priming the animals with an agonist of TLR3, TLR4, or TLR9 enhances survival to *F. tularensis* infection and decreases organ bacterial burdens (33–35).

Recent studies have indicated that Kdo residues contribute to proinflammatory responses to LPS (36–38). Therefore, the presence of a side chain Kdo in the $\Delta kdhAB$ mutant, with direct recognition of this modified LPS by the TLR2 signaling pathway, has emerged as a potential mechanism for the observed phenotype. However, our *in vitro* studies suggest that this is not the case, as purified LPSs from the $\Delta kdhAB$ mutant and LVS elicit similarly weak NF- κ B activation (Fig. 4B). Another possible explanation is that the structural alteration of LPS exposes one or more TLR2 ligands present on the bacterial surface. The electrostatic charge of LPS is important for the folding of certain outer membrane proteins (39–42), and Kdo—as a negatively charged sugar—contributes to the overall charge of LPS. Thus, the presence of an additional Kdo residue in $\Delta kdhAB$ mutant LPS may alter the conformation of the outer membrane, exposing TLR2 ligands such as lipoproteins (43). In support of this idea, we showed that FopA surface protein is more accessible to antibodies in the $\Delta kdhAB$ mutant than in parental LVS (Fig. 5B).

LVS is an attenuated *F. tularensis* subsp. *holarctica* strain (type B) that was empirically derived in the former Soviet Union and developed as a live attenuated vaccine against the highly virulent *F. tularensis*. However, the LVS-based vaccine remains unlicensed in the United States and several other nations for several reasons, including the following: (i) an incomplete understanding of the genetic basis for its attenuation; (ii) untoward reactions; and (iii)

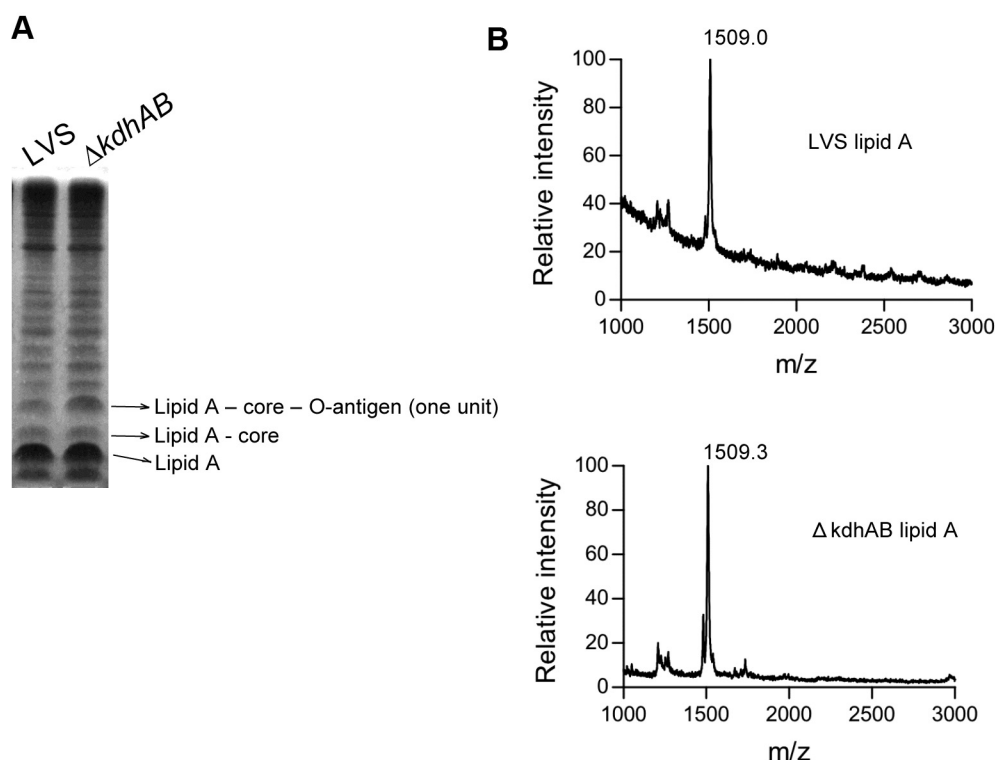


FIG 6 The *F. tularensis* $\Delta kdhAB$ mutant strain is not altered in the biosynthesis of O antigen or lipid A. (A) SDS-PAGE analysis of *F. tularensis* LVS and $\Delta kdhAB$ mutant outer membranes. LPS was visualized by zinc staining. (B) Negative-ion MALDI-TOF analysis of lipid A from LVS and $\Delta kdhAB$ bacteria.

concerns about possible reversion back to the virulent form. The mechanisms of LVS-mediated protection against tularemia involve TLR2-dependent innate immunity (25–27). Therefore, an LVS mutant that exhibits attenuated virulence in mice and more strongly elicits activation of TLR2 signaling might prove to be a safer and more effective vaccine candidate than the original LVS. Here we show that the $\Delta kdhAB$ LVS mutant may be such a vaccine candidate: its virulence is attenuated, its ability to activate TLR2 is enhanced, and its protective efficacy against *F. tularensis* type A strain Schu S4 is similar to that of LVS. Mutants unable to modify lipid A by carbohydrate incorporation were attenuated in mice, activated proinflammatory responses, and were protective against *F. tularensis* subsp. *novicida* (8). However, protection against the highly virulent *F. tularensis* type A strain Schu S4 has not been investigated. Few previously assessed mutants have been shown to induce protective immunity against type A strains (for a review, see reference 44). These mutants have had deletions in genes including *FTT1103*, *FTT0918*, *sodB*, *wzy*, and *ggt* (45, 46). We report a new mutation that is relevant for tularemia vaccine design.

TABLE 1 Survival of WT C57BL/6, TLR2^{−/−}, and TLR4^{−/−} mice 21 days after an intranasal challenge with *F. tularensis* $\Delta kdhAB$ mutant

Challenge dose (CFU)	Survival rate (%) of mice:		
	C57BL/6 (n = 10)	TLR2 ^{−/−} (n = 10)	TLR4 ^{−/−} (n = 5)
10 ²	100	100	100
10 ³	100	40	100
10 ⁴	100	0	100

Herein we demonstrate that the two-component Kdo hydrolyase KdhAB is involved in the virulence of *F. tularensis* LVS. A $\Delta kdhAB$ mutant is attenuated in mice, elicits a stronger proinflammatory cytokine response in a TLR2-dependent manner, and induces protective immunity against the highly virulent Schu S4 strain of *F. tularensis*. Our data suggest that a novel LPS modification—the removal of side chain Kdo—is important in the evasion of innate immunity by *F. tularensis*. Kdo hydrolase is also produced by two other pathogens, *H. pylori* and *L. pneumophila* (14, 15, 17, 18). It will be particularly interesting to study the role of this enzyme in the virulence of these organisms.

MATERIALS AND METHODS

Ethics statement. All work with animals was conducted according to the recommendations in the *Guide for the Care and Use of Laboratory Animals* (47). The protocol was approved by the Standing Committee on Animals of the Harvard Medical Area (protocol 04723).

Bacterial strains and plasmids. *Francisella tularensis* LVS (kindly provided by Karen Elkins, U.S. Food and Drug Administration, Bethesda, MD) was grown in cysteine heart agar supplemented with 1% hemoglobin (CHAH) for 72 h at 37°C and 5% CO₂, in modified Muller-Hinton broth (Becton, Dickinson, Franklin Lakes, NJ) supplemented with ferric pyrophosphate and IsoVitaleX (Becton, Dickinson, Franklin Lakes, NJ), or in tryptic soy broth (Difco, Detroit, MI). *Escherichia coli* strain DH5 α was used for general cloning and was grown either in Luria-Bertani (LB) broth or on LB agar plates. When appropriate, ampicillin (100 μ g/ml) and kanamycin (50 μ g/ml) were added to broth medium, and kanamycin (10 μ g/ml) was added to CHAH.

Construction of the *F. tularensis* LVS $\Delta kdhAB$ deletion mutant. The $\Delta kdhAB$ mutant was constructed by allelic exchange (48) by a procedure described previously (14). The whole *kdhA* gene was deleted. As the last

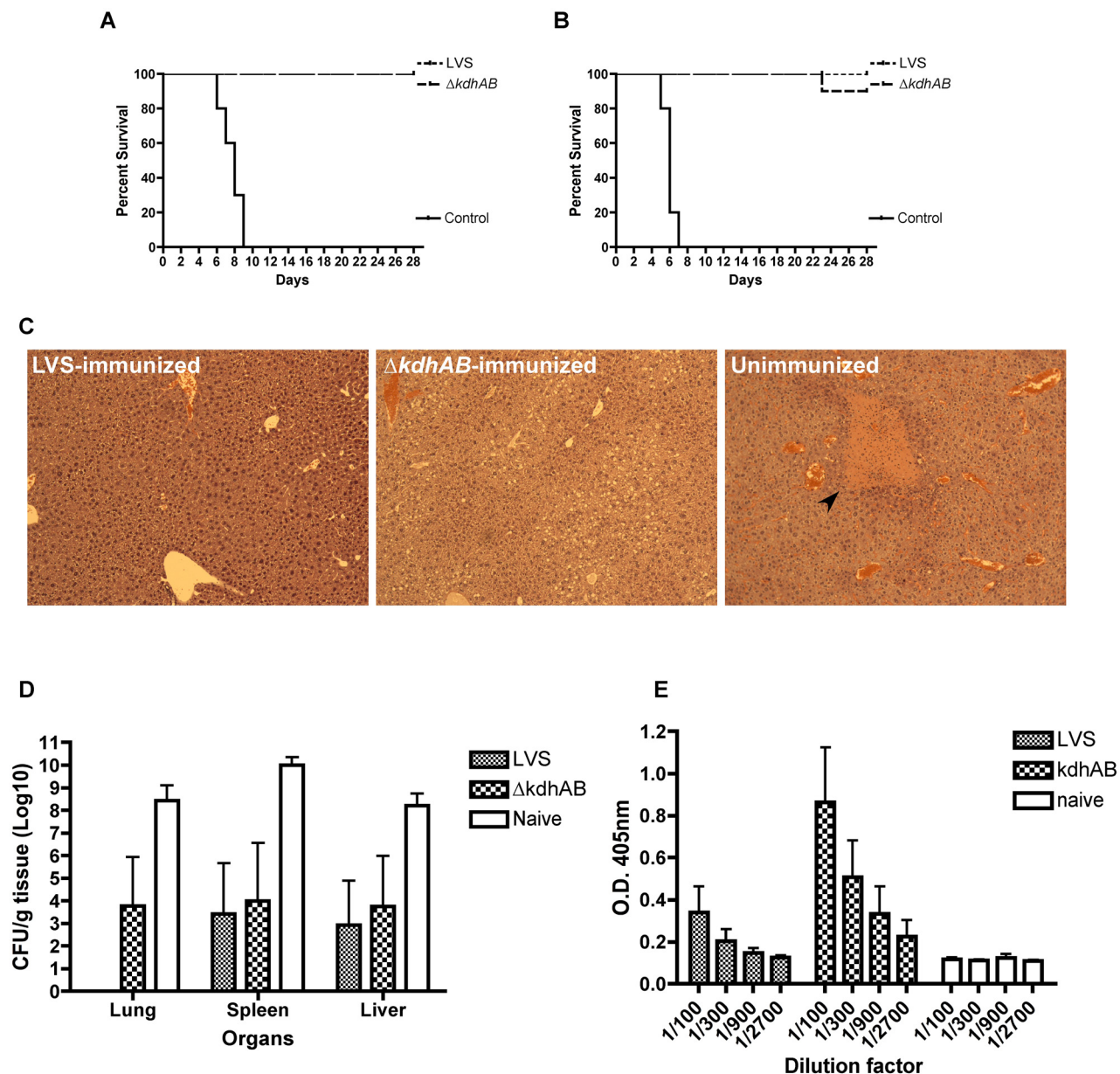


FIG 7 Bacteria of the *F. tularensis* LVS $\Delta kdhAB$ mutant strain elicit protective immunity against *F. tularensis* LVS and type A strain Schu S4. (A and B) Groups of BALB/cByJ mice (10 mice in each group) were immunized intranasally with 3 doses of LVS or $\Delta kdhAB$ bacteria; 28 days after the last dose, the mice were challenged intranasally with either 10^4 CFU of LVS ($10 \times LD_{50}$) (A) or 10 CFU of strain Schu S4 ($10 \times LD_{50}$) (B). (C) Hematoxylin and eosin (H&E) staining of liver tissue on day 5 after Schu S4 challenge. The black arrowhead indicates extensive tissue necrosis ($10\times$). (D) Bacterial burden in lung, spleen, and liver of mice 5 days after Schu S4 challenge (mean plus SD; $n = 3$). (E) Titers of serum antibody to *F. tularensis* LPS were determined by ELISA. Sera from LVS-immunized, $\Delta kdhAB$ mutant-immunized, and unimmunized control mice were collected on the day before challenge. ELISA was performed as described in Materials and Methods.

eight bases of *kdhA* overlap with *kdhB*, the first eight bases of *kdhB* were also deleted. The following primers were used: 5'-ACCCCGGGCACTTTCGTGCTTAGATATCT-3' (XmaI site underlined) and 5'-TGTGGTACAGTTTTAAAAATTAAGTAAGT-3' (KpnI site underlined) to amplify the 1-kb upstream region; 5'-ACCGGTACCTAATTATAACCTTTTGAC AAC-3' (KpnI site underlined) and 5'-TGTGAATTTCGAAAGTAGGTGATATGATTGC-3' (EcoRI site underlined) to amplify the 1-kb downstream region.

For complementation studies, plasmid *pkdhAB* encoding *kdhAB* genes under a *groEL* promoter was constructed. In brief, a DNA sequence

containing open reading frames of both *kdhA* and *kdhB* genes was amplified by PCR with primers 5'-TGGAAATTCATGAAACATAAACTAAAGCTAG-3' (EcoRI site underlined) and 5'-TCCTCGAGTTAGTAATATATAATCTTATCTT-3' (XhoI site underlined). After restriction endonuclease digestion, the *kdhAB* DNA fragment was ligated into the EcoRI and XhoI sites of plasmid pTH17 (49).

Bacterial DNA/RNA preparation. Plasmids were introduced into *F. tularensis* LVS by electroporation, and miniplasmids for *E. coli* DH5 α and *F. tularensis* LVS were prepared with a QIAprep spin miniprep kit according to the manufacturer's instructions (Qiagen, Venlo, Nether-

lands). Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs. DNA ligations and transformations of chemically competent *E. coli* were performed by standard protocols (50).

LPS purification. LPS was purified from *F. tularensis* WT and $\Delta kdhAB$ strains as described elsewhere (49). A modification of the hot phenol-water method was used (51).

Mouse infection and immunization studies. Male BALB/cByJ, C57BL/6, TLR2^{-/-}, and TLR4^{-/-} specific-pathogen-free mice (6 to 8 weeks old; Jackson Laboratory, Bar Harbor, ME) were housed in an animal facility at Harvard Medical School. All procedures conformed to protocols approved by the Institutional Animal Care and Use Committee. Bacterial virulence was assessed by intranasal challenge of BALB/cByJ mice ($n = 6$) with 10^3 CFU of *F. tularensis* LVS bacteria or with 10^3 , 10^4 , or 10^5 CFU of $\Delta kdhAB$ bacteria (inoculum volume, 50 μ l per mouse). Mice were lightly anesthetized with isoflurane (Baxter, Deerfield, IL). The importance of the TLR2 signaling pathway in mouse survival after intranasal challenge with $\Delta kdhAB$ mutant bacteria (10^2 , 10^3 , or 10^4 CFU) was assessed in C57BL/6 WT, TLR2^{-/-}, and TLR4^{-/-} mice. In all these instances, survival was monitored over a 26-day period unless otherwise indicated.

For immunization studies, groups of BALB/cByJ mice ($n = 10$) were immunized by the intranasal route with three doses of bacteria at 2-week intervals; the doses were 10^3 , 10^4 , and 10^5 CFU for *F. tularensis* LVS and 10^3 , 10^4 , and 10^5 CFU for the $\Delta kdhAB$ mutant. A mock immunization group that received PBS served as a control. Four weeks after the last dose, mice were challenged intranasally with either 10^4 CFU of LVS ($10 \times \text{LD}_{50}$) or 10 CFU of strain Schu S4 ($10 \times \text{LD}_{50}$). Mice were challenged with the *F. tularensis* Schu S4 strain at the animal biosafety level 3 (ABSL3) core facility at Harvard Medical School. Titers of serum antibody to LPS were measured by ELISA as described previously (26).

Organ bacterial burden and histopathology. BALB/cByJ mice were challenged intranasally with 10^3 CFU of *F. tularensis* LVS or $\Delta kdhAB$ bacteria. Six days after infection, mice were killed, blood was collected, and tissues were weighed, hand mashed, and homogenized with a Stomacher 80-paddle action blender (Seward, Port Saint Lucie, FL). Serial 10-fold dilutions were prepared with sterile $1 \times$ PBS supplemented with 2% fetal bovine serum. A 10- μ l volume of each dilution was plated onto CHAH plates and incubated at 37°C in 5% CO₂ until colonies became visible (~72 h). Data were expressed as CFU per gram of tissue or per milliliter of blood.

Histological analysis was performed on the livers of *F. tularensis* Schu S4-challenged mice that had been immunized with *F. tularensis* LVS or $\Delta kdhAB$ mutant or had been given PBS as a control. Livers from each group of mice were fixed in 10% formalin solution, embedded in paraffin, and stained with hematoxylin and eosin after sectioning.

Bronchoalveolar lavage. BALB/cByJ mice were challenged intranasally with 10^3 CFU of *F. tularensis* LVS or $\Delta kdhAB$ bacteria. After 24 h, the mice were killed, the tracheae were exposed, and the lungs were lavaged with 0.5 ml of PBS supplemented with 0.5 mM EDTA. TNF- α and IL-1 β were assayed with ELISA DuoSet kits (R&D Systems, Minneapolis, MN).

BMM infection and measurements of cytokine production and gene expression. Bone marrow was flushed with PBS from the femurs of C57BL/6J mice (6 to 8 weeks old; Jackson Laboratory), spun down, resuspended in BMM growth medium (Dulbecco modified Eagle medium [DMEM] containing D-glucose [4.5 mg/liter], L-glutamine [4 mM], and sodium pyruvate [110 mg/liter] [Invitrogen, Carlsbad, CA] supplemented with 10% heat-inactivated fetal bovine serum [FBS] [Invitrogen] and 30% cell line L929-conditioned DMEM with nonessential amino acids). The cells from each femur were plated in two deep petri dishes, and the plates were incubated at 37°C in 5% CO₂ in the presence of gentamicin (50 μ g/ml). After 3 days, BMM growth medium was added to petri dishes. The cells were incubated for an additional 2 to 3 days.

For measurements of cytokine release, BMMs were seeded in 24-well plates at a density of 5×10^5 cells/well, and the plates were incubated at

37°C in 5% CO₂ overnight. The macrophages were then infected with mid-logarithmic-phase *F. tularensis* LVS or $\Delta kdhAB$ bacteria at an MOI of 10, 20, or 50 (ratio of bacteria to macrophages) for 24 h. TNF- α and IL-1 β in cell supernatants were measured with ELISA DuoSet kits according to the manufacturer's recommendations (R&D Systems).

For measurements of gene expression, BMMs were seeded in 6-well plates at a density of 2×10^6 cells/well, and the plates were incubated at 37°C in 5% CO₂ overnight. The macrophages were then infected with mid-log *F. tularensis* LVS or $\Delta kdhAB$ bacteria at an MOI of 10. At various time points, cells were lysed in 0.4 ml TRIzol (Invitrogen), and RNA was isolated according to the manufacturer's instructions. Reverse transcription was performed with a qScript Flex cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's instructions. Real-time PCR was conducted with Perfecta SYBR green supermix (Quanta Biosciences) in a Bio-Rad iCycler iQ system (45 cycles, with 1 cycle consisting of 15 s at 95°C, 45 s at 60°C, and 30 s at 72°C). Gene expression was normalized to the expression of the RNA polymerase II (RP II) gene, which is considered a stably expressed housekeeping gene (52). The following primer sets were used: for TNF- α , sense (5'-TATGG CTCAGGGTCCAATC-3') and antisense (5'-CTCCCTTTGCAGAACT CAGG-3'); for IL-1 β , sense (5'-GGGCCTCAAAGGAAAGAATC-3') and antisense (5'-TACCAGTTGGGGAACCTGC-3'); and for RP II, sense (5'-GCACCACGTCCAATGATAT-3') and antisense (5'-CTGGCGGTT GACCCCATGAC-3'). Relative gene expression was calculated by the $\Delta\Delta C_T$ method (53) and reported as fold induction over the uninfected control level (mean \pm standard deviation [SD] values for samples from three independent experiments).

Murine macrophage-like cell line infection. WT, Unc93B^{-/-}, MyD88^{-/-}, TLR4^{-/-}, and TLR2^{-/-} murine macrophage-like cell lines, kindly provided by Douglas T. Golenbock (University of Massachusetts Medical School, Worcester, MA), were seeded in 24-well plates at a density of 5×10^5 cells/well, and the plates were incubated overnight at 37°C in 5% CO₂. The macrophages were then infected with mid-log *F. tularensis* LVS or $\Delta kdhAB$ bacteria at an MOI of 100. After 2 h, cells were washed three times with Dulbecco's PBS and lysed in 60 μ l of lysis buffer (kit BF14100; R&D Systems). IL-1 β in lysates was measured with an ELISA DuoSet kit (R&D Systems).

NF- κ B reporter luciferase assay. Stable HEK293 cell lines expressing TLR2 receptor were kindly provided by Douglas T. Golenbock. HEK-TLR2 and HEK-TLR4/MD2 cells were seeded into 12-well tissue culture plates at a density of 3×10^5 cells/well. The following day, cells were transiently transfected with luciferase reporter genes; Genejuice (EMD Chemicals, Darmstadt, Germany) was used per the manufacturer's recommendations. To assess NF- κ B activation, an NF- κ B luciferase reporter gene (consisting of an artificial promoter NF- κ B site driving the firefly luciferase gene) was cotransfected with a constitutively active *Renilla* luciferase reporter gene, phRL-TK (Promega, Fitchburg, WI); phRL-TK was used to normalize transfection efficiency. The following day, the cells were stimulated as indicated in Results. During stimulation with LPSs from *F. tularensis* LVS or $\Delta kdhAB$ bacteria, the mixture contained soluble CD14 recombinant protein (5 μ g/ml) (ProSci Inc., Poway, CA). After 24 h of stimulation, the cells were lysed in passive lysis buffer (Promega), and reporter gene activity was measured with a plate reader luminometer (Victor2; PerkinElmer Life Sciences) and the Dual-Luciferase reporter assay system (Promega).

Surface antigen-binding assay. Evaluation of FopA antibody binding to the bacterial surface was performed as described previously (24), with some modifications. In brief, cultures of WT *F. tularensis* LVS, $\Delta kdhAB$, and $\Delta wbtA$ bacteria were grown overnight in brain heart infusion (BHI) broth. Cultures were diluted in fresh BHI medium to an optical density at 620 nm (OD₆₂₀) of 0.07, and bacteria were further grown to an OD₆₂₀ of 0.5. After the bacteria were harvested, 6×10^8 bacteria from each strain (in a 50- μ l volume) were mixed with monoclonal FopA antibodies (final concentration, 0.1 mg/ml) (54) in PBS containing 0.1% glucose and 0.5% bovine serum albumin (BSA). After incubation for 1 h at 37°C, bacteria

were washed three times with PBS containing 0.1% glucose. After the final wash, pellets were resuspended in Laemmli sample buffer (Bio-Rad, Hercules, CA). Protein samples were resolved on 5 to 20% Protean TGX gels (Bio-Rad) and transferred to nitrocellulose membranes. Alkaline phosphatase (AP)-conjugated rabbit anti-mouse antibody (Abcam, Cambridge, MA) was used to detect heavy- and light-chain subunits of bound FopA antibodies.

LPS analysis by SDS-PAGE and MALDI-TOF mass spectrometry. Outer membranes from *F. tularensis* LVS and $\Delta kdhAB$ bacteria were isolated, resolved by Tricine SDS-PAGE, and stained with zinc as previously described (14). Lipid A from LVS and $\Delta kdhAB$ bacteria was isolated and analyzed by negative-ion matrix-assisted laser desorption/ionization—time of flight (MALDI-TOF) mass spectrometry (14).

Data analysis. Data are expressed as mean \pm standard deviation (SD) values. Unpaired Student's *t* test was used for statistical analysis. A *P* value of <0.05 was considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00638-12/-/DCSupplemental>.

Figure S1, EPS file, 0.8 MB.

Figure S2, JPG file, 0.5 MB.

Figure S3, JPG file, 0.5 MB.

Table S1, DOCX file, 0.1 MB.

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